Intracellular chitinase gene from *Rhizopus oligosporus*: molecular cloning and characterization

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Multiple chitinases have been found in hyphae of filamentous fungi, which are presumed to have various functions during hyphal growth. Here it is reported, for the first time, the primary structure of one such intracellular chitinase, named chitinase III, from *Rhizopus oligosporus*, a zygomycete filamentous fungus. Chitinase III was purified to homogeneity from actively growing mycelia of *R. oligosporus* using three steps of column chromatography. Its molecular mass was 43.5 kDa and the pH optimum was 6.0 when *p*-nitrophenyl *N,N,N'*- β -D-triacetylchitotrioside was used as a substrate. Chitinase III also hydrolysed chromogenic derivatives of chitobiose, but had no *N*-acetylglucosaminidase activity. The gene encoding chitinase III (*chi3*) was cloned using PCR with degenerate oligonucleotide primers from the partial amino acid sequence of the enzyme. The deduced amino acid sequence of *chi3* was similar to that of bacterial chitinases and chitinases from mycoparasitic fungi, such as *Aphanocladium album* and *Trichoderma harzianum*, but it had no potential secretory signal sequence in its amino terminus. Northern blot analysis showed that *chi3* was transcribed during hyphal growth. These results suggest that chitinase III may function during morphogenesis in *R. oligosporus*.

Keywords: *Rhizopus oligosporus*, zygomycetes, chitinase

INTRODUCTION

Chitin, a homopolymer of 1,4- β -linked *N*-acetyl-D-glucosamine, is widely distributed in invertebrates, fungi and algae and confers strength against mechanical forces from the environment as external skeletons or cell walls. In filamentous fungi, chitin is one of the major components of the cell wall and is thought to be important for maintaining cell wall integrity. Proper chitin metabolism may be essential for hyphal growth and morphology.

Chitinase (EC 3.2.1.14) is an enzyme which hydrolyses chitin at 1,4- β -bonds between *N*-acetylglucosamine

units. The enzyme is found in bacteria, plants, insects, humans and fungi (Flach *et al.*, 1992; Collinge *et al.*, 1993; Boot *et al.*, 1995) and is thought to function in assimilation of chitin, defence against fungal pathogens and separation of dividing cells, although the actual roles of most chitinases remain to be elucidated. In filamentous fungi, chitinases are thought to be involved in processes requiring cell wall digestion (Gooday, 1990), viz. germination of spores, tip growth of hyphae (Bartnicki-Garcia, 1973), branching of hyphae, hyphal autolysis and differentiation into spores, as well as in assimilation of chitin and mycoparasitism. There have been few reports of chitinases of filamentous fungi. In *Mucor rouxii*, multiple chitinase activities have been observed in mycelial extracts (Rast *et al.*, 1991) and germinating spores (Pedraza-Reyes & Lopez-Romeo, 1991). Their roles in fungal growth and mechanisms of chitinase regulation are almost totally unknown.

Rhizopus oligosporus is a filamentous fungus that belongs to the phylum Zygomycota, whose cell walls are mainly composed of chitin and its deacetylated de-

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Abbreviation: PNP, *p*-nitrophenyl.

The DDBJ accession number for the *chi3* sequence reported in this paper is D87894.

rivative, chitosan (Davis & Bartnicki-Garcia, 1984). Because of the abundance of chitin and chitosan, *R. oligosporus* could be a good organism for the analysis of cell wall chitin metabolism. We have already cloned two chitin synthase genes (Motoyama *et al.*, 1994) and also two chitinase genes, *chi1* and its allelic gene *chi2*, encoding chitinase I and chitinase II, respectively, from this organism (Yanai *et al.*, 1992). In the course of our study, it was revealed that chitinase I and chitinase II are induced in the late stage of culture, when the hyphae autolyse, so that we could purify the enzymes from the culture medium. It was also found that they are synthesized with N-terminal signal sequences for secretion. These results suggest that chitinase I and II are not involved in hyphal growth.

With the aim of revealing the function of chitinases in growing hyphae, we report here the purification and characterization of chitinase III from mycelial extracts of *R. oligosporus* and the cloning of the gene encoding this enzyme. Our results suggest that chitinase III has a role during hyphal growth and that filamentous fungi have evolutionarily divergent chitinases with markedly differing roles in chitin metabolism.

METHODS

Strains and media. *R. oligosporus* IFO8631 was used throughout this work. *R. oligosporus* was grown in SIV (Takaya *et al.*, 1994) or potato dextrose medium. *Escherichia coli* MV1190 [$\Delta(lac-proAB)$ *thi supE* $\Delta(srl-recA)$ 306::Tn10 Tet^r (F' *traD36 proAB lacI*^qZ Δ M15)] was used for the propagation of plasmids. *E. coli* was grown in LB medium and 50 μ g ampicillin ml⁻¹ was added if necessary.

Culture conditions. Surface culture was done as described previously (Yanai *et al.*, 1992). Submerged culture was done as follows: spores (approx. 8×10^7) of *R. oligosporus* were inoculated into a 5 litre Erlenmeyer flask containing 1 litre SIV medium and cultivated in a rotary shaker at 120 r.p.m., 30 °C.

Enzyme activity assay. Chitinase assay was performed with the chromogenic substrate *p*-nitrophenyl *N,N',N''*- β -D-tri-acetylchitotrioside (PNP chitotrioside; Seikagaku-Kogyo). Reactions contained 0.1 M potassium citrate (pH 6.5), Triton X-100 (0.1%, v/v), 0.5 mM PNP chitotrioside at 37 °C in a total volume of 100 μ l. The reaction was stopped with 500 μ l 1 M Na₂CO₃ and the absorption of *p*-nitrophenol was spectrophotometrically determined at 415 nm (Hitachi U-2000 spectrophotometer). One unit of enzymic activity was defined as that which released 1 nmol *p*-nitrophenol min⁻¹.

Enzyme purification. Chitinase I was purified as described previously (Yanai *et al.*, 1992). For purification of chitinase III, 12 litres SIV medium was inoculated with 10⁹ spores of *R. oligosporus* and incubated with shaking at 30 °C for 18–20 h. Mycelia (130 g wet wt) were harvested by filtration, washed with 20 mM NaHCO₃ (pH 8.4) and suspended in 600 ml of the same buffer containing 1 mM EDTA, 1 mM PMSF and 10 mM β -mercaptoethanol. Mycelia were broken with a Waring blender at top speed. Triton X-100 was added to a final concentration of 1% and the broken mycelial mixture stirred for 3 h at 4 °C. After centrifugation at 7000 *g* for 30 min, the supernatant was loaded onto a regenerated-chitin column (16 $\times$ 70 mm) and washed with 600 ml 20 mM

NaHCO₃ (pH 8.4) containing Triton X-100 (0.1%) at a flow rate of 50 ml h⁻¹. Fractions containing chitinase activity that were eluted by 20 mM sodium acetate (pH 3.2) containing Triton X-100 (0.1%) were immediately neutralized with 1 M Tris. They were combined (105 ml), dialysed against 20 mM Tris/HCl (pH 8.0) containing Triton X-100 (0.1%) and applied to a DEAE-Sephadex A50 column (Pharmacia) (10 $\times$ 100 mm). The column was washed with 100 ml of this buffer at a flow rate of 50 ml h⁻¹ and chitinase activity eluted with a linear gradient from 0 to 500 mM NaCl in 300 ml of the same buffer. Fractions (84 ml) containing chitinase activity were pooled, dialysed against 1 mM sodium phosphate, 0.1% Triton X-100 (pH 6.8) and loaded onto a hydroxyapatite (Wako Chemicals) column (10 $\times$ 60 mm). After washing with 100 ml 1 mM NaCl, 40 mM octyl D-glucoside (Dojindo), chitinase activity was eluted with 1 mM sodium phosphate (pH 6.8), 40 mM octyl D-glucoside. Fractions containing chitinase activity were pooled, dialysed against 40 mM octyl-D-glucoside and used as the purified chitinase III preparation. This preparation was used for all enzyme characterization studies with chitinase III.

Subcellular fractionation. Subcellular fractionation of *R. oligosporus* was done as follows: 0.5 g (wet wt) of mycelia of *R. oligosporus* cultured in SIV medium containing glucose as carbon source was suspended in 5 ml 50 mM sodium phosphate (pH 7.2) and broken with a Potter-Elvehjem homogenizer. Cell extracts were centrifuged at 1000 *g* for 15 min and the supernatant was recentrifuged at 10000 *g* for 15 min at 4 °C to prepare the 10000 *g* pellet. The supernatant was centrifuged at 100000 *g* for 1 h at 4 °C to obtain the 100000 *g* pellet and 100000 *g* supernatant.

Solubilization of proteins. Solubilization of the 10000 *g* and 100000 *g* pellets was carried out as follows: 10000 *g* or 100000 *g* pellets containing approximately 20 mg protein were suspended in 50 mM sodium phosphate (pH 7.2) to a final volume of 4 ml. Triton X-100 was added to a final concentration of 1% and the resuspension incubated for 1 h at 4 °C. It was then centrifuged at 10000 *g* for 15 min at 4 °C. The supernatants were loaded directly onto the chitin column.

SDS-PAGE and Western blot analysis. SDS-PAGE was done as described by Laemmli (1970). Western blot analysis was performed with ECL-Western blotting detection reagents (Amersham) according to the manufacturer's instructions.

Protein sequencing. *In situ* digestion of chitinase III was done essentially according to the method of Aebersold *et al.* (1987). Chitinase III (about 60 μ g) was subjected to SDS-PAGE and transferred by blotting to nitrocellulose membrane (Schleicher & Schuell). The protein bands were detected by staining with Ponceau S (Sigma) and a protein of 43.5 kDa excised and digested with trypsin. The tryptic fragments were separated by C18 reverse-phase HPLC and the amino terminal sequences of three of these fragments (P15, P32 and P34) determined using an automated amino acid sequencer (Applied Biosystems model 373A).

Isolation of the *chi3* gene. *R. oligosporus* total DNA, prepared as described previously (Horiuchi *et al.*, 1988), was used as a template for PCR with the following degenerate oligonucleotide primers: 31, 5' GCCATNCCNACNAC 3' (corresponding to the sequence of tryptic peptide P32); and 32, 5' TTYGCNAAYGTCNARCC 3' and 33, 5' ATRTCNGCR-TANGCRTC 3' (both corresponding to the sequence of tryptic peptide P34. N, Y and R indicate any of the four nucleotides, a pyrimidine and a purine, respectively). The first PCR was done with primer 31 (100 pmol), primer 32

(100 pmol) and 0.5 µg total DNA as a template. Cycling conditions were: 10 min at 94 °C for the first denaturation; 30 cycles of 0.5 min at 94 °C, 1 min at 42 °C and 2 min at 72 °C for amplification; then 10 min at 72 °C for extension. Products of about 700 bp were purified by agarose gel electrophoresis and used as a template for the second PCR. The second PCR was performed using the same conditions except for the use of primers 32 and 33. An amplified DNA fragment of the expected size was obtained and purified. Labelled probes were prepared using [α -³²P]dCTP by the random primer labelling method (Feinberg & Vogelstein, 1983) and used for screening of a genomic library constructed in vector λ 2001 (Yanai *et al.*, 1992) by plaque hybridization (Sambrook *et al.*, 1989).

Nucleotide sequencing. A 3.8 kb *Sall*–*Pst*I fragment bearing the *chi3* gene was inserted into pUC119 and a series of deletions generated using exonuclease III and mung bean nuclease. The truncated *chi3* DNAs were sequenced by the dideoxy chain-termination method (Sanger *et al.*, 1977). In some cases, restriction fragments of the *chi3* DNA were cloned into pUC119 and sequenced. Primer extension analysis was done as described by Rothmel & LeClerc (1989) using the oligonucleotide 5' CAGGTCCGTTTGCTGCTGGAGGTG-GTTCC 3' as a primer.

RNA analysis. Spores of *R. oligosporus* (10^5 ml⁻¹) were cultured in SIV medium containing glucose (2%, w/v) at 30 °C for 24 h. Mycelia were then transferred to SIV medium containing no carbon source, 0.1% glucose, 2% glucose, or 10% (w/v) colloidal chitin (Yanai *et al.*, 1992) and incubated at 30 °C for 24 h. Total RNA was prepared from the mycelia using the RNeasy Total RNA kit (Qiagen) according to the manufacturer's instructions. Thirty micrograms of each RNA preparation were separated by agarose gel electrophoresis, blotted to Hybond-N (Amersham) and hybridized with the [α -³²P]-labelled *chi3* cDNA fragment as a probe. Signals were detected using a Bioimaging Analyzer BAS2000 (Fuji Photo Film).

RESULTS

Purification of chitinase III

To purify intracellular chitinase in the present study, we used actively growing hyphae of *R. oligosporus* as starting material, in contrast to our previous work (Yanai *et al.*, 1992). Mycelial homogenates were extracted with buffer containing Triton X-100 (1%) and the soluble fraction was subjected to a series of column chromatography procedures. During the first regenerated-chitin column chromatography step, significant activity was eluted as a single peak with 20 mM acetate (pH 3.2) containing Triton X-100 (0.1%) and was subjected to further purification. As no activity was eluted with the buffer lacking Triton X-100, the detergent was included in the buffer in the following steps. During DEAE-Sephadex chromatography, the enzyme activity was eluted as a broad peak from 100 mM to 400 mM NaCl concentration. This step had little effect on purification, but was helpful for concentrating the enzyme activity in the following final step. By the procedure described, we purified the chitinase activity by 2400-fold with a yield of 1.3% from the detergent-soluble fraction of mycelia. SDS-PAGE of this fraction showed a single band of molecular mass of 43.5 kDa

Fig. 1. Analysis of protein profiles during chitinase III purification procedure. Approximately 30 µg protein (lane 1) or 2 µg protein (lanes 2–5) were separated on a 10% SDS-PAGE gel and visualized by Coomassie brilliant blue staining. Molecular mass standards are indicated in kDa. Lanes: 1, crude 1% Triton X-100 extract; 2, 20 mM sodium acetate eluate from regenerated-chitin column; 3, pool from DEAE-Sephadex A50 column; 4, pool from hydroxyapatite column; 5, purified chitinase I.

(Fig. 1, lane 4) and we designated this protein chitinase III. A summary of the representative purification steps is shown in Table 1.

Characterization of the purified enzyme

The isoelectric point of chitinase III was approximately 7.0 (data not shown). No positive signal was obtained by periodic acid/Schiff reagent staining (Poehling & Neuhoﬀ, 1981) after SDS-PAGE (data not shown). This was consistent with the lack of consensus sequences for asparagine-linked glycosylation in the deduced amino acid sequence (see below), suggesting that chitinase III has no glycosyl chain. The optimum pH for enzyme activity with PNP chitotriptide as substrate was approximately 6.0, which is much higher than that for chitinase I (optimum pH 3.0), another chitinase purified from this fungus (Yanai *et al.*, 1992) (data not shown). The specific activity for PNP chitotriptide was 19.8 U (mg protein)⁻¹, which was 8.9-fold lower than that of chitinase I. The specificity of each chitinase for chromogenic chito oligoside substrates was determined. The activity of chitinase III with PNP chitobioside or PNP chitotetraoside as substrate was approximately one-fifth and sevenfold that for PNP chitotriptide, respectively, whilst the activity of chitinase I with PNP chitobioside or PNP chitotetraoside as substrate was below one-fiftieth and 10-fold that for PNP chitotriptide, respectively (experiments were undertaken three times with the same results, data not shown). K_m values of chitinase III and chitinase I for PNP chitotriptide were 7.8×10^{-5} M and 1.3×10^{-4} M, respectively. The K_i of chitinase III for allosamidin, a competitive inhibitor of chitinases, was 3.0×10^{-8} M, which was one-sixth that of chitinase I (1.8×10^{-7} M). $MnCl_2$ (1 mM) increased the activity of

Table 1. Purification of chitinase III from mycelial extract of *R. oligosporus*

Data from a typical purification procedure are presented. Three independent isolation experiments gave comparable results.

Purification step	Protein (mg)	Total activity (U)	Specific activity [U (mg protein) ⁻¹]	Yield (%)	Purification (-fold)
Crude extract	7728	64000.0	8.28	100	1.0
Regenerated chitin	1.56	5950.0	3814.1	9.3	460.6
DEAE-Sephadex	0.80	1182.1	1477.5	2.0	178.4
Hydroxyapatite	0.043	853.3	19844.2	1.3	2396.6

chitinase III 2.7-fold, but had no effect on chitinase I. The metal ion chelator (10 mM EDTA) had no effect on chitinase III activity, but inhibited chitinase I activity by approximately 50%. The activities of these chitinases were not affected significantly by DTT (10 mM), β -mercaptoethanol (10 mM), $MgCl_2$ (1 mM), $CaCl_2$ (1 mM), $ZnCl_2$ (1 mM), $NaMoO_4$ (1 mM) or $CoCl_2$ (1 mM) (data not shown).

A preliminary result suggested that the activity of chitinase III increased in the presence of Triton X-100 (0.1%) and this may be indicative of association with other macromolecules or membranes. Therefore, we examined the effects of the detergent, a phospholipid and serum albumin on enzyme activity. The results showed that activity of the purified enzyme increased approximately fourfold in the presence of each of Triton X-100 (0.1%), serum albumin (1 $\mu g\ ml^{-1}$) or phosphatidylcholine (1 $\mu g\ ml^{-1}$) (data not shown).

Intracellular localization of chitinase III

We investigated the distribution of chitinase III in *R. oligosporus* by Western blot analysis of a few subcellular fractions using antibody, raised against *Bacillus circulans* chitinase A1, which did not react with chitinase I or II. Using this antibody, only a single protein band of 43.5 kDa was detected following Western blot analysis of the fraction, eluted from the chitin column during enzyme purification, that contained chitinase III activity. We added Triton X-100 to each of the 10000 g and 100000 g pellet fractions (final concentration, 1%) to solubilize the pellet (see Methods section), purified chitinase activity from these and the 100000 g supernatant fraction using the regenerated-chitin column, and carried out Western blot analysis with anti-chitinase A1 antiserum (Fig. 2). Chitinase III was detected in both 100000 g supernatant and 10000 g pellet fractions. These results suggest that some portion of chitinase III is associated with membrane- or cell-wall structures in *R. oligosporus*.

Amino acid sequencing of chitinase III

Direct sequencing of the amino-terminal amino acids of chitinase III was unsuccessful. Therefore, three tryptic peptides of chitinase III (P15, P32 and P34) were sequenced. The amino acid sequences of P15, P32 and

Fig. 2. Western blot analysis of subcellular fractions of *R. oligosporus* lysate. Approximately 1.0 μg protein per lane was transferred to nitrocellulose and incubated with anti-chitinase A1 antiserum. Lanes: 1, solubilized 10000 g pellet; 2, solubilized 100000 g pellet; 3, 100000 g supernatant.

P34 were YNVVDVATQANK, LVVGMPYGR and AFANVQPDGQVVLGDAYADI, respectively. Searching the protein databases (SWISS-PROT and PIR), we found that peptide P32 had similarity to chitinase 1 of *Aphanocladium album* (7 of 10 amino acid positions were identical) (Blaiseau & Lafay, 1992) and chitinase A1 of *B. circulans* (8 of 10 amino acid positions were identical) (Watanabe *et al.*, 1990). Peptide P34 also had similarity to the *A. album* chitinase (10 of 20 amino acid positions were identical), whilst peptide P15 showed no significant homology to known sequences.

Cloning of the chitinase-III-encoding gene

To clone the gene encoding chitinase III, nested PCR (McPherson *et al.*, 1995) was performed with three degenerate oligonucleotide primers based on the sequences of peptides P32 and P34. The nucleotide sequence of a specifically amplified 0.11 kb fragment was determined and its deduced amino acid sequence completely matched the sequence of P34, if we assumed

Fig. 3. Primer extension analysis of *R. oligosporus chi3*. (a) Lanes A, C, G and T indicate profiles of DNA sequences assayed by the dideoxy method. Lane P, products of primer extension reaction. Transcription start sites are shown by dots on the left. (b) Transcription start sites and the translation initiation site are indicated by dots and box, respectively.

an intron between the fourteenth amino acid (glycine)- and fifteenth amino acid (aspartic acid)-encoding sequences. A genomic DNA library was screened using this DNA fragment as a hybridization probe and four positive clones were obtained. They were divided into two classes by their restriction maps. A 3.8 kb *SalI*–*PstI* fragment from one of the positive clones was inserted into pUC119 and its nucleotide sequence was determined. One ORF containing three putative intron sequences was present and its deduced amino acid sequence contained sequences that matched peptides P15, P32 and P34.

Since the transcription start site was at the twenty-seventh T, as determined by the primer extension method (Fig. 3a), the translation initiation site was

Fig. 4. Northern blot analysis using *chi3* cDNA as hybridization probe. (a) *R. oligosporus* was cultured in SIV medium containing glucose as carbon source for 24 h at 30 °C, then transferred to SIV medium containing one of the following carbon sources: none (lane 1); glucose (0.1%) (lane 2); glucose (2%) (lane 3); colloidal chitin (10%) (lane 4) and incubated for 24 h at 30 °C (Yanai *et al.*, 1992). RNA was isolated as described in the text and 30 µg RNA electrophoresed per lane. (b) Total RNA was prepared from submerged culture in SIV medium containing glucose (2%) as sole carbon source after 24 h (lane 1), surface culture on the same medium after 24 h (lane 2) and surface culture after 48 h (lane 3). RNA was isolated and electrophoresed as described above. Positions of rRNAs are shown on the left in kb.

assigned as shown in Fig. 3(b). The presence of the introns was confirmed by the sequence of the cDNA of *chi3* (data not shown). These results indicated that this DNA fragment contained the chitinase-III-encoding gene, which was designated *chi3*. The predicted amino acid sequence was comprised of 400 amino acids and its calculated molecular mass was 44.9 kDa, which corresponds well to that of chitinase III. There is neither a potential secretory signal sequence in its amino terminus nor a long hydrophobic stretch in the internal sequence. The partial nucleotide sequence of another clone with a different restriction map was also determined. Its deduced amino acid sequence was very similar to that of chitinase III (data not shown), suggesting that this clone could contain an allele of *chi3*, which is consistent with the observation that *R. oligosporus* is diploid or aneuploid (Motoyama *et al.*, 1994; Yanai *et al.*, 1992).

Homology to other chitinases

Although there is no potential secretory signal sequence, the amino acid sequence of chitinase III is similar to a number of sequences reported previously for chitinases, demonstrating 42, 42, 42, 42, 40 and 25% similarity to *A. album* chitinase 1 (Blaiseau & Lafay, 1992), *Trichoderma harzianum* ThEn-42 chitinase (Hayes *et al.*, 1994), *T. harzianum* ECH-42 chitinase (Carsolio *et al.*, 1994), *T. harzianum* CHIT42 chitinase (García *et al.*, 1994),

Coccidioides immitis CTS1 chitinase (Pishko *et al.*, 1995) and *B. circulans* chitinase A1 (Watanabe *et al.*, 1990), respectively. *A. album* chitinase 1 and *T. harzianum* ECH-42 chitinase have been reported to be similar to bacterial chitinases. On the other hand, chitinase III has very limited similarity to yeast chitinases (Kuranda & Robbins, 1991; McCreath *et al.*, 1995) or to chitinase I and chitinase II of *R. oligosporus* (Yanai *et al.*, 1992), except for short conserved sequences (+101 LSIIGG +105 and +139 DGLDIDWE +146; the numbers represent amino acid residues from the amino terminus of chitinase III), which are known to be important for catalytic activity (Watanabe *et al.*, 1993). These results indicate that chitinase III belongs to the bacterial-type chitinase subclass rather than to the fungal-type chitinase subclass of yeasts and filamentous fungi.

Expression of *chi3* mRNA in *R. oligosporus*

Northern blot analysis was performed using *chi3* cDNA as a hybridization probe. *chi3* mRNA was abundant in actively growing cells and was not increased by addition of colloidal chitin (Fig. 4a). It was reduced when the mycelia were transferred to a medium which contained only a low concentration of carbon or no carbon source. Furthermore, *chi3* mRNA was not detected during the late stage of surface culture (Fig. 4b, lane 3), under conditions where chitinase I was produced in abundance (data not shown), indicating that the expression of *chi3* and of *chi1* is regulated differently during growth.

DISCUSSION

In this study, we have purified and characterized intracellular chitinase III from the mycelial extract of *R. oligosporus*, cloned its encoding gene and analysed the structure of the gene. This is the first report of the isolation of the gene encoding an intracellular chitinase from a filamentous fungus. The optimum pH for enzyme activity was nearly neutral and higher than that of extracellular chitinase I, suggesting that chitinase III may function in the cytosol. The deduced amino acid sequence of chitinase III had no potential secretory signal sequence and no possible sequence for modification by carbohydrate chains commonly found in secretory proteins. Interestingly, its deduced amino acid sequence indicates that it belongs to the bacterial-type chitinase subgroup rather than the fungal-type subgroup, in spite of the fact that the bacterial-type chitinases reported so far have hydrophobic residues in their amino termini and are known to be extracellular proteins. It is possible that there is another intracellular chitinase in *R. oligosporus* which does not bind to the chitin column under our experimental conditions since a substantial part of the total chitinase activity was detected in the unbound fraction during chitin column chromatography.

Chitinase III exhibited a higher affinity than chitinase I for small chitooligosides. Chitinase I has a serine/threonine-rich domain and a chitin-binding domain on the carboxy terminus, which have been suggested to act

as acceptor sites for O-glycosylation and high-affinity chitin binding, respectively (Kuranda & Robbins, 1991). In contrast, chitinase III had no such domains. This difference may be related to the varying substrate specificity of the two enzymes and to the different chitinase subgroups they belong to, since the bacterial-type chitinase (Chit42) from *T. harzianum* cleaves smaller oligomers of chitin than the fungal-type chitinase (Chit33) (De la Cruz *et al.*, 1992).

In the subcellular fractionation experiment, significant amounts of chitinase III were detected in the 10000 g pellet, which could be solubilized in the presence of Triton X-100, and the activity of this enzyme was stimulated by Triton X-100, serum albumin and phospholipid. These results suggest that chitinase III could be most active in the cells in contact with membrane- or cell-wall structures. In *M. rouxii* and *Candida albicans* cell extracts the presence of membrane-bound chitinases is suggested and the activities of these enzymes are inhibited by treatment with phospholipases and stimulated by detergents (Humphreys & Gooday, 1984; Dickinson *et al.*, 1991). A 45 kDa chitinase was purified from the cytosolic fraction of *C. albicans*. The enzyme has an optimum pH of 5.0 and chitinase activity was stimulated 1.5-fold when phosphatidylcholine was present in the reaction mixtures (Mellor *et al.*, 1994). These characteristics are similar to those of chitinase III studied during the present investigation. We are interested in the possibility that the 45 kDa chitinase of *C. albicans* is a bacterial-type chitinase, similar to those detected in filamentous fungi, which has not so far been detected in yeasts. In this regard it is important to note that *C. albicans* is a dimorphic fungus capable of filamentous growth.

The expression pattern of *chi3* suggests that the enzyme is produced during hyphal growth. Chitinases are thought to be involved in hyphal tip growth and branching. It is possible that chitinase III may function in loosening the cell wall at the hyphal tip to enable turgor pressure to extend the hypha at the apex (Bartnicki-Garcia, 1973). Rast *et al.* (1991) proposed a model for functional relationships between chitinases and chitin synthases. According to this model, there are two types of chitinases in mycelia of *M. rouxii*. The type A chitinases, which hydrolyse smaller chitooligosides efficiently, are proposed to provide N-acetylglucosamine to activate chitin synthase(s) allosterically. The other, type B chitinases, are speculated to provide substrates for type A chitinases by hydrolysing long-chain cell wall chitin. Our results suggest that chitinase III hydrolyses well small chitooligosides rather than large ones and could interact with membrane- or cell-wall structures in the cell, proposals which are compatible with the ideas of Rast *et al.* (1991) described above. Stimulation of chitinase activity by Mn^{2+} , as observed during the present study, has also been reported for some chitin synthases (Sudoh *et al.*, 1995).

Recently, both fungal- and bacterial-type chitinase genes were cloned from each of two fungi: *T. harzianum* (Carsolio *et al.*, 1994; García *et al.*, 1994; Hayes *et al.*,

1994; Limón *et al.*, 1995) and *C. immitis* (Pishko *et al.*, 1995). We also cloned both types of chitinase genes (*chiA* and *chiB*) from *Aspergillus nidulans* (Takaya *et al.*, 1998; unpublished results). In *T. harzianum*, the expression of Chit42 is induced by chitin in the culture medium. In contrast, *R. oligosporus* chitinase I (unpublished results) and chitinase III are not induced by chitin. It is probable that the genes encoding these two different types of chitinase evolved before these fungi diverged from their common ancestor, and that differing gene-regulating mechanisms have evolved in different fungi living in different environments.

ACKNOWLEDGEMENTS

We are grateful to Dr S. Sakuda and Dr T. Watanabe for providing allosamidin and anti-chitinase A1 antiserum, respectively. We are also grateful to Dr S. Nakajo and Dr K. Nakaya for the amino acid sequencing analysis of chitinase III.

This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan. This work was performed using the facilities of the Biotechnology Research Center, The University of Tokyo.

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Received 24 March 1998; revised 7 May 1998; accepted 14 May 1998.